ABSTRACT

CYNTHIA D. HANSON Evaluation of the Effect of Low Level 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Exposure on Cell Mediated Immunity. (Under the direction of RALPH J. SMIALOWICZ)

The immunotoxicity of TCDD in the mouse has been well documented. To date, the most sensitive endpoint of TCDD-induced immunotoxicity in mice is that reported by Clark et al.(Proc.Soc.Exp.Biol.Med. 168:290, 1981) who found that TCDD suppressed the murine cytotoxic T lymphocyte (CTL) response at levels as low as 4ng/kg. However, these data have not been corroborated, as other laboratories have been unable to detect immunosuppression by TCDD at such low levels. In this study, we evaluated the effect of TCDD on the in vivo and in vitro generated CTL response to P-815 mastocytoma cells in adult C57BL/6J female mice via a ^{51}Cr release assay. Mice were given weekly intraperitoneal injections of TCDD or vehicle for four weeks at doses ranging from 0.01 to 3.00 µg/kg/week. No statistically significant suppression of the in vivo or in vitro CTL response was detected at any dose. As expected, significant increases in liver weights and decreases in thymus weights were observed at TCDD doses of 1.0 and 3.0 µg/kg/week. Likewise, suppression of the antibody plaque-forming cell response to sheep erythrocytes was suppressed at doses of 1.0 and 3.0 µg/kg/week TCDD. Though expected humoral immunosuppression and organ effects were observed, our data do not support suppression of murine CTL responses at the TCDD doses employed in this study.

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I. INTRODUCTION - TCDD

2,3,7,8 Tetrachlorodibenxo-p-dioxin (referred to herein as TCDD) has been called the most toxic compound known to man. Despite inconclusive data in humans, TCDD invariably results in alteration of the gamut of biological processes in laboratory animals. This report presents a review of the literature on TCDD and evaluates results of the effect of TCDD on cell mediated immunity.

Dioxin (specifically dibenzodioxin) is a general name for a family of compounds. The name is indicative of the basic structure - two benzene rings bridged by two oxygen molecules. The molecule may be chlorinated (or otherwise halogenated) at any of eight available sites to form a chlorinated dioxin. The volatility and solubility are inversely proportional to the degree of chlorination (Skene et al., 1989); the biological activity of the molecule is also influenced by chlorination. Maximum activity requires that no fewer than three and preferably four of the 2,3,7,8 positions be chlorinated. Seventy-five possible congeners exist, of which the 2,3,7,8 tetrachlorodibenzo-p-dioxin is the most biologically active and the most intensely studied.

Except in small amounts for research purposes, TCDD is not manufactured. It is an undesirable by-product formed during the production of 2,4,5-trichlorophenol (TCP) from 1,2,4,5 tetrachlorobenzene (Homberger et al., 1979). TCP is produced as a precursor to the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and to hexachlorophene, a local antiseptic for gram negative bacteria (Travis and Hattemer-Frey, 1991). Original levels of TCDD

contamination of 2,4,5-T ranged from 1 to 70 ppm; the Council of Scientific Affairs reported in 1982 that this contamination has been regulated at less than 0.01 ppm. The infamous herbicide Agent Orange consisted of a 50:50 mixture of 2,4,5-T and 2,4 dichlorophenoxyacetic acid (2,4-D) and was contaminated with varying amounts of TCDD. For decades, mixtures of 2,4-D and 2,4,5-T have been used by homeowners and farmers for herbicidal purposes (Council on Scientific Affairs, 1982). Other sources of TCDD include municipal waste and hospital incinerators, diesel and leaded gasoline burning engine emissions and paper mill effluent (Skene et al., 1989; Travis and Hattemer-Frey, 1991). Skene et al. (1989) report that even cigarette smoke and char-broiled steaks contain low levels of TCDD.

TCDD is ubiquitous throughout the environment. It has been measured in all media studied including air, soil, meat, milk, fish, vegetation and human adipose tissue (Travis and Hattemer-Frey, 1991). It is present in virtually all human adipose tissue at levels greater than 3 ppt (Travis and Hattemer-Frey, 1991) and normal background body levels in North America range up to 20 ppt (Gough, 1991). Gough (1991) notes that men aged 70 years or over, having only ever lived in the isolated western desert, far from any industry, have adipose tissue levels of 6 to 7 ppt.

Once in the environment, the bioavailability of TCDD is limited except through the food chain. It is strongly held by soil, relatively immobile (Homberger et al., 1979; Skene et al., 1989) and is transported by wind and rain only so far as its bound particle (Tschirley, 1986). Since TCDD is lipophilic, it bioaccumulates; the food chain is thus the major route of uptake (Travis and Hattemer-Frey,

1991). Dermal absorbtion and inhalation of contaminted particles account for other routes of exposure especially following industrial or environmental accident.

TCDD is persistant in the environment when protected from light but breaks down rapidly in sunlight (Tschirley, 1986). Photolysis degrades TCDD in the presence of proton donors, such as oils on the surface of plant leaves and in moist soils (Council on Scientific Affairs, 1982; Homberger et al., 1979). The half life of TCDD in surface soils has been estimated at 230 days (Council on Scietific Affairs, 1982) and up to 10 years in areas protected from sunlight (Tschirley, 1986). Microorganisms can degrade TCDD, but do so slowly (Tschirley, 1986).

The toxicity of TCDD (and similar halogenated hydrocarbons or HAH's) is believed to be mediated through an intracellular protein receptor, the Ah receptor. This receptor was originally found in murine liver cells and has been found in almost all tissues studied (Greenlee et al., 1991). The ubiquitous presence of the Ah receptor suggests that other mechanisms must influence the extent of responses mediated by the receptor (Greenlee, 1988; Greenlee et al., 1991). The receptor is thus considered necessary but not sufficient for induction of TCDD toxicity.

Induction results from the initial, reversible binding of TCDD to the receptor and subsequent transduction of the TCDD-receptor complex to the nucleus, where it is able to bind specific DNA recognition elements and alter gene expression (Neal, 1985). Credence is given to the theory that TCDD toxicity is necessarily mediated through the receptor due to its solitary lack of covalent binding to genetic material and negative results in short-term genotoxic bioassays.

There exists little human data to suggest that TCDD exerts severe adverse effects. TCDD has not been implicated as the cause of even one human death and most epidemiological studies are inconclusive. Much of this data is from incidents surrounding the spraying of massive amounts of herbicide in Vietnam, the ICMESA plant accident in Seveso, Italy, and the spraying of TCDD contaminated oils in and around Times Beach, Missouri.

Problems exist in studying human exposure to chemicals. First, defining the exposed population (Young, 1984) and determining the level of exposure accurately are extremely difficult. Also, singling out a compound as wide-spread as TCDD as the direct cause of any effect is nearly impossible. This problem is exacerbated by the fact that TCDD rarely occurs in the environment alone, but in conjunction with other potentially hazardous compounds.

The one consistent outcome of TCDD exposure in humans is the development of chloracne. This affliction appears two weeks to two months following exposure and ususally clears 8 - 26 months after exposure ceases, the length of affliction depending on the severity of exposure (Reggiani and Hoffman, 1980). However, there has been no correlation found between chloracne and alteration in other biological functions such as increase in serum enzymes and adverse neurological findings (Homberger et al., 1979; Reggiani and Hoffman, 1980). No difference between exposed and non-exposed groups have been observed including damage to blood cells, kidney function, reproductive outcomes and liver function, or an increase in chromosome abberations (Reggiani and Hoffman, 1980; Webb, 1984; Homberger et al., 1979). In terms of immunological function, no suppression of cell mediated immunity (Stehr

et al., 1986) or lymphoproliferation to mitogens (Stehr et al., 1986; Hoffman, 1986) was observed. However, some alteration, though not statistically significant, has been seen in T lymphocyte subpopulation ratios (Hoffman et al.,1986; Stehr et al., 1986; Evans et al., 1988).

The U.S. Environmental Protection Agency is currently re-evaluating its regulation of TCDD. Due to lack of human evidence, the EPA has conservatively set the current Acceptable Daily Intake (ADI) for TCDD at 6 fg/kg/day, using a linear, multistage model. This model assumes a linear relationship exists between outcome and exposure, that adverse effects begin with exposure to a single molecule and progress, and that no adverse effects threshold exists (Roberts, 1991b). Using a No Observable Effect Level (NOEL) and incorporating safety factors, the Canadian Ministry of the Environment, along with some European countries, have set their ADI at 10 pg/kg/day, a level 1670 times that in the U.S.. Recent regulators have been cautiously suggesting that, if indeed receptor binding is the essential first step in TCDD toxicity, then this implies that there is a threshold or NOEL below which no adverse effect occurs and that current EPA regulations are too strict (Roberts, 1991a, 1991b; Greenlee et al, 1991).

There exists a respectable amount of data in the literature on the immunotoxic effects of TCDD, including a 1981 paper by Clark et al.. The paper reports the most sensitive endpoint to TCDD mediated immunotoxicity to date. Here, suppression of the cytotoxic T lymphocyte (CTL) response was detected at levels as low as 4 ng/kg. The work has been controversial because not only have the data never been corraborated but all other reports suggest that TCDD begins exerting its adverse effects on the immune system at the microgram per kilogram

level or higher. Such a paper may present a problem to the re-evaluation of TCDD regulation. The purpose of this study was to repeat Clark's method and re-evaluate the effect of TCDD on the CTL response.

II. IMMUNOLOGY REVIEW

The immune system has evolved as an extremely complex and highly specific network of organs and cells aimed at ridding its host of foreign invaders. Since the number and type of possible antigens is vast, so too is the variability with which this system is able to attack. Thus, cells of the immune system undergo highly controlled stages of differentiation before giving rise to such funtionally mature cells as granulocytes, macrophages and lymphocytes (Dean, 1986). Depending on the tissue in which maturation occurs, lymphocytes are generally classified as T-cells or B-cells; T-cells are thymus dependent, whereas mammalian B-cells mature in the bone marrow.

Two general types of immunity are recognized - innate and specific. Innate immunity is nonspecific and requires no prior contact with foriegn agonist; it lacks specificity for any particular antigen. Such a non-specific system is useful to any organism as a ready defense against antigens such as bacteria, viruses and fungi. Non-specific immunity is most often attributed to both the phagocytic activity of neutrophils, monocytes and macrophages, (Barrett, 1988) and the cytolytic activity of natural killer (NK) cells.

Specific immunity refers to that which is developed over the lifetime of an organism. It is characterized by the development of immunity for a particular antigen and of immune "memory" for that antigen in preparation for subsequent exposure. It is antigen specific

and based on either the activities of soluble antibody (humoral immunity, HI) or cell mediated immunity (CMI). HI and CMI are usually attributed to B- and T-lymphocytes, respectively, though the two populations rarely operate independently of one another.

Perturbations of the immune system, by environmental chemicals or otherwise, may be detrimental to the host. An altered immune system may be antagonistic to its host, as in the cases of allergy and autoimmunity. Chemical exposure may render an organism more susceptible to disease due to alterations in host defense mechanisms and immunosuppression. The work presented here examines the immunosuppressive capabilities of TCDD on aquired immunity, specifically on the cytotoxic T-lymphocytes.

At the core of immunity, is the requirement that host lymphocytes must recognize antigen. All cells express gene products encoded for by a group of genetic loci termed the Major Histocompatibility Complex (MHC). These protein antigens allow for distinction between self and non-self by acting as cell surface markers. MHC antigens are able to induce a sub-population of T-cells that are capable of lysing target cells against which they have been primed, the cytotoxic T lymphocytes (CTL). There is disagreement surrounding the actual mechanism by which CTL lyse their target cells. However, it is known that CTL kill autonomously (Nabholz and MacDonald, 1983), that the lytic event requires actual contact between CTL and its target (Nabholz and MacDonald, 1983) and that following delivery of the lethal hit, the CTL is able to recycle and interact with more targets (Berke, 1991).

III. IMMUNOTOXICITY LITERATURE REVIEW

The biological and toxicological effects of TCDD are characterized by diversity. Species differences in site of accumulation, LD_{50} values as well as age differences have been well documented. Retention of tritiated TCDD in the liver accounted for over 40% of the administered dose in rats, while liver retention in monkeys was less than 10% (VanMiller et al., 1975). In these monkeys, a large percentage of this TCDD was found in high lipid content tissues such as skin, muscle and fat. Gasiewicz et al. (1983) used tritiated TCDD, at a dose of 10µg/kg, in the C57BL/6J, DBA/2J and B6D2F1/J strains of mice to determine the liver and adipose tissue as major sites for accumulation. However, even intraspecies differences exist. The higher percent adipose tissue in the DBA/2J mice versus the other two strains accounts for slower excretion rate in these mice.

The lethal dose for 50% of a population (LD_{50}) is a crude, yet widely used measure of toxicity and is especially applicable when making comparisons. The LD_{50} values for TCDD vary widely among rodents. Guinea pigs have been shown to be the most sensitive; hamsters are among the least (see Table 1).

Toxicity induced by TCDD is more profound in the young and unborn than in adult animals. Thymus, spleen and body weights were depressed at birth in pups of mothers treated with TCDD during gestation (Faith and Moore, 1977; Vos and Moore, 1974). Vos and Moore (1974)

demonstrated the weight response to be dose dependent, the higher dose of 5µg/kg administered to the mother resulting in large numbers of stillbirths. Ninety-one percent of the mouse pups died by 25 days when treatment via nursing mothers was continued into the postnatal period. These effects occured far below lethal doses for adult mice. Pre- and postnatal treatment exposure of rats has greater and longer lasting effects than postnatal treatment alone (Faith and Moore, 1977). Recovery of body weight was never complete in groups treated pre- and postnatally. Splenic and thymic responses to mitogens (Concanavalin A and phytohemagglutinin) were always suppressed compared to those exposed only postnatally and to controls. Also found to be suppressed in pups of treated mothers were cell-mediated immunity (CMI) (Moore and Faith, 1976), graft versus host (GVH) reactions (Vos and Moore, 1974), and the ability to mount delayed-type hypersesitivity (DTH) reactions against oxazolone (Faith and Moore, 1977).

Organ weight effects, however, are a hallmark of TCDD exposure. Thymic atrophy and decreased in cellularity are usually severe (Chastain and Pazdernik, 1985; Moore and Faith, 1976) and have been shown to be dose dependent (Fine et al. 1990; Vos and Moore, 1974). Likewise, atrophy of the spleen, though not as sensitive to TCDD as the thymus, has been shown to be dose dependent (Vos and Moore, 1974). An increase in liver weight is routinely noticed following TCDD exposure. This effect also, has been shown to be dose dependent by Kerkvliet et al. (1990).

Effects on organ weights are suggestive of toxicity to that organ. Thus, consistent thymic and splenic atrophy prompted research on the immunotoxic effect of TCDD. Generally speaking, the mode through which

TCDD exerts its immuntoxicity is unclear and there is disagreement concerning the type of immunity most senstive to TCDD. Most immunological parameters have been looked at and several have been suggested as a target for TCDD toxicity. Vos and Moore (1974) pinpointed the thymus as a target organ. Naturally, this suggests T-cells and therefore cell-mediated immunity as targets for TCDD induced toxicity. This is supported by Moore and Faith (1976) who found CMI suppression in offspring of TCDD treated animals and claim CMI to be more sensitive to TCDD toxicity than humoral immunity. Clark et al. (1981) have reported suppression of CTL response as the most sensitive indicator of toxicity; however, the doses they used are extremely low and the results have never been duplicated. Chastain and Pazdernik (1985) also claim CMI to be more sensitive than B-cell suppression upon exposure to TCDD. Vecchi et al., (1980) reported no decrease in ability to mount graft versus host (GVH) reactions.

The B-cell has also been suggested as the target lymphocyte for TCDD toxicity. The antibody forming cell response, or plaque forming cell (PFC) response is a standard assay for assessing humoral immunity and is consistently suppressed in mice upon treatment with TCDD. Suppression of this response in mice to both T-dependent and T-independent antigens begins to appear at around 1.0 µg/kg (House et al. 1990; Vecchi et al. 1980, 1983; Davis and Safe, 1990). Lymphoproliferation to mitogen stimulation is suppressed following in utero exposure (Faith and Moore, 1977) but not in exposed adult mice (Vecchi et al., 1980). Using standard assays for T helper cell population using mitogen stimulated cells, Dooley et al. (1990) found no significant difference in tritiated thymidine uptake or in

interleukin-2 production. They also found that TCDD treated T-cells were not able to suppress naive splenocyte humoral response to the T-dependent antigen, sheep red blood cells (SRBC) or the T-independent antigen (DNP-Ficoll). Such results suggest the TCDD induced alterations in T-cell function play no role in suppressing the antibody response to antigen. Tucker et al. (1986) also noted that TCDD affects the differentiation of B-cells into antibody secreting cells and thus concluded the B-cells as target for TCDD.

TCDD suppression of innate immunity has been looked at by White et al. (1986), who showed decreased levels of serum complement component C3 at levels of TCDD as low as 0.5 µg/kg in B6D2F1 mice. No significant difference in natural killer cell or macrophage function has been obseved (House et al., 1990; Mantovanni, 1980). Some alterations in host resistance observed are increased suseptibiblity to Streptococcus pneumonia (White,1986) and increased mortality due to Influenza virus (House et al. 1990) and Herpes Simplex II virus (Clark et al. 1983). No increase in mortality due to Listeria monocytogenes following TCDD exposure was found by House et al. (1990).

Increase in liver weight is indicative of TCDD-induced hepatotoxicity. TCDD exposure results in enzyme induction such as aryl hydrocarbon hydrolase (AHH) (Tucker et al., 1986). A variey of enzymes are temporarily induced by TCDD including Cytochrome P-450 (monooxygenase), glutathione-s-transferase, UDP-glucuronyltransferase, and choline kinase (Neal, 1985). Many compounds able to induce AHH are also immunosuppressive (Tucker et al., 1986) and induction of AHH has been shown to correlate with the toxicity of TCDD and other halogenated aromatic hydrocarbon (HAH) congeners (Vecchi et al., 1983). The

correlation between toxicity and enzyme induction is mediated by the Ah receptor, which has a high binding affinity for TCDD and similar HAH's. Low binding affinity correlates with reduced sensitivity to TCDD toxicity (Clark et al., 1983).

The Ah locus codes for the Ah receptor. Strains of mice that differ at the Ah locus also differ in their response to TCDD. For example, C57B1/6 mice (Ah^b), because of their sensitivity to TCDD have been designated Ah-responsive while DBA/2 mice (Ah^d) are more resistant and designated Ah-nonresponsive. Studies suggest that TCDD toxicity segregates with the Ah locus (Tucker et al., 1986) including suppression of the PFC response (Davis and Safe, 1990) and CTL suppression (Clark et al., 1983). Kervliet et al. (1990) looked at C57B1/6 mice congenic at the Ah locus (Ah^{bb} and Ah^{dd}) to incontestibly demonstrate that sensitivity to TCDD correlates with the Ah locus and not with other differences between strains. The CTL response was suppressed in both strains at 10µg TCDD/kg and 20µg TCDD/kg, however suppression in the Ah^{bb} was significantly and dramatically greater. The same held true for reduction in thymus weight. Also, compared in this study were three hexachlorobiphenyl (HxCB) congeners of known affinity (high, intermediate, and low) for the Ah receptor. CTL response, thymic weight reduction and corticosteroid induction correlated with the affinity of the compounds.

IV. The assays

The ^{51}Cr release assay is a classic quantitation of lytic capability (Henney, 1971; Brunner, 1968). Therefore, it lends itself well to the study of CTL and the effect of chemical exposure on their fidelity. The assay relies on sensitized CTL recognizing and effectively lysing labelled target cells. Lymphocytes are sensitized with foreign (i.e., allogeneic) cells in vivo or lymphocytes in vitro. The splenic lymphocytes or effector cells are then plated against the original tumor (or target) cells labelled with ^{51}Cr. Viable CTL lyse the target cells, releasing ^{51}Cr into the supernatant, which is collected and quantified as a measure of cytotoxic efficiency. It is important to note that the assay measures lytic activity of populations of cells; it does not enumerate the number of cytotoxic cells in a given population (Nabholz and MacDonald, 1983). Generally, the following relationship is considered for conversion of raw counts to a relative percent release scale:

$$(E-S)/(T-S) \times 100\%$$

where

E= release from spleen and target cells

S = spontaneous release (targets only)

T = maximum release (targets + 1% Triton-X)

The plaque-forming cell (PFC) assay is widely used to assess antibody formation at the cellular level (Barrett, 1988). Splenocytes are plated in nutrient agar containing erythrocytes of the type used during prior immunization. PFC quickly secrete antibody against erythrocytes, which subsequently become coated with the antibody. The additon of serum complement to the medium promotes the lysis of the red blood cells, leaving behind a clear sphere, or plaque around an antibody forming lymphocyte. The plaques are enumerated as a measure of immunological capability.

The PFC assay obviously evaluates humoral immunity. However, the immunosuppressive activity of TCDD has been well studied in this laboratory using the PFC assay. Therefore, it was employed in this study as a benchmark for immunosuppression and insurance of dosing procedure.

V. MATERIALS

Animals

C57BL/6J adult female mice from Jackson Laboratories, Bar Harbor, ME, were employed throughout this study. The animals were housed 6-10 per cage containing heat-treated pine shavings (Beta Chips, North Eastern Products Inc., Warrensberg, NY) in a controlled environment room with a 12 hour light/dark cycle at an ambient temperature of 22±^{o}C and 50±5% relative humidity. They were allowed food (Purina Lab Chow, Ralston Purina Co., St. Louis, MO) and water ad libitum.

Cell lines

The P-815 mastocytoma cell line was carried both in sterile tissue culture and by weekly serial passage by ascites in DBA/2J adult female mice (Jackson Laboratories, Bar Harbor, ME) housed as described above. P-815 cells in tissue culture were maintained in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 25mM HEPES, and 1% penicillin-streptomycin. Sterile Costar 50 ml tissue culture flasks (Cambridge, MA) were seeded twice weekly and incubated at 37^{o}C, 5% CO_2.

Chemical Exposure

TCDD (Radian Corporation, Austin, TX) was dissolved in acetone and subsequently diluted in corn oil. The C57BL/6J mice were given either a single intraperitoneal (ip) dose, at a dose rate of 10ml/kg or dosed weekly for four weeks. Control animals were given corn oil alone.

Sensitization

Spleen cells were allo-sensitized to either P-815 cells or DBA/2J spleen cells (both H-2^d) one week following the final TCDD dose either by in vivo or in vitro method. For in vivo sensitization, the animals were immunized, ip, with $2x10^7$ P-815 cells (suspended in approximately 3 ml RPMI-1640 media). The mice were allowed to mount an immune response for 10-11 days, after which the spleens were removed for the assay.

To sensitize spleen cells in vitro, a mixed lymphocyte reaction (MLR) was established. When two histoincompatible lymphocyte populations are co-cultured, T-cell proliferation and CTL differentiation ensue (Henney and Gillis, 1984). Usually, a one-way reaction is engaged, wherein the proliferation of one cell type is prevented. Here, DBA/2J spleen cells were irradiated at 2000 rad using a Cesium source (National Institute of Environmental Health Sciences, RTP, NC) to render them incapable of replication. Spleens were aseptically removed and a single cell suspension prepared using a Stomacher Lab-Blender (80) (Tekmar, Cinncinnati, OH) and Seward Medical

Stomacher sterile bags. All in vitro MLR experiments were carried out in RPMI-1640 media supplemented with 10% FBS, 25 mM HEPES, 0.1% gentamycin and $5x10^{-5}$ M 2-mercaptoethanol. Cell counts were taken on a Coulter Counter Model ZBI (Coulter Electronic Inc., Hialeah, FL) and viabilities determined using the trypan blue exclusion method. The C57BL/6J spleen cells were plated as responder cells (R) in a one way MLR against the stimulator (S) irradiated DBA/2J spleen cells at R:S ratios = 50:1 or 20:1 depending on cell yield. The Costar 6-well plates were incubated at $37^{o}C$, 5% CO_2 for 5 days.

For the PFC assay, mice were immunized, intravenously (iv), with 0.2ml of 5% SRBC four days prior to removal of spleen for assay.

VI. METHODS

For the ^{51}Cr assay, P-815 cells were collected from ascites, using RPMI-1640 media + 25mM HEPES and centrifuged at 250 x g for 7 minutes. After counts and viabilities were determined, three times the calculated number of cells needed for labelling were aliquoted. ^{51}Cr at 10mCi/ml was added at 10µl/1x10^6 cells and incubated for one hour. Cells were then centrifuged and washed three times in assay media. For the purpose of lowering background counts as much as possible, the cells were resuspended and incubated for another hour. Following incubation, the cells were centrifuged, resuspended in assay medium, counted using a hemacytometer and resuspended to a final concentration (1x10^5 cells/ml in vivo, 2x10^5 cells/ml in vitro).

Spleen cells were either collected from the 6-well plates or taken from the sensitized animals and processed as described above. Media for the ^{51}Cr assay consisted of RPMI-1640 supplemented with 10%FBS, 25mM HEPES, and 0.1% gentamycin. Final concentrations for cell suspensions equal to 1x10^7 ml in vivo, 4x10^6 ml in vitro. In 96-well U bottomed tissue culture plates (Costar, Cambridge, MA), spleen cells were plated as effector cells (E) against ^{51}Cr labelled P-815 target cells (T) at E:T ratios = 100, 50, 25, 12.5:1 in vivo and 20, 10, 5, 2.5:1 in vitro. Plates were centrifuged at 250 x g for 5 minutes and placed in a 37°C, 5%CO_2 incubator for four hours. After incubation plates were removed and centrifuged a second time, the supernatant was

collected using Skatron macrowell filters (Skatron, Inc.,Sterling, VA). The amount of radioactivity in the supernatant fraction was determined using a Packard Multi-Prias gamma counter (Packard Instrument Co., Downers Grove, IL).

For the PFC assay, the spleens from immunized animals were removed and processed as described above using RPMI-1640 media supplemented with 5% FCS and 0.1% gentamycin. The single cell suspension was allowed to settle for 10 minutes and then passed through a type II, class 2, cheese cloth filter (The Kendall Company, Boston, MA) to remove debris. Dilutions of the suspension were made in media at 1:20 and 1:40. Sample cell counts were determined as described above.

SRBC (Environmental Diagnostics, Inc., Burlington, NC) were washed three times with saline and resuspended to 40%. Reconstitued guinea pig complement (Gibco, Grand Island, NY), previously absorbed with one drop of washed SRBC and frozen, was thawed and diluted 1:3 with assay media. Agar was prepared using 0.5% Bacto-Agar (Difco, Detroit, MI) and 0.05% diethyl aminoethyl cellulose (DEAE-dextran, Pharmacia Fine Chemicals, Uppsala, Sweden) in Earles's balanced salt solution (EBS). The agar was dissolved in EBS in a boiling water bath, DEAE was then added and the agar solution was maintained at 47^{o}C in a constant temperature water bath.

The spleen cells, agar, SRBC, and complement (20%, 5%, 70%, and 5%, respectively) were added to 10 x 75 mm glass tubes in duplicate. The solutions were vortexed briefly and poured into 100 mm petri plates (Costar, Cambridge, MA) and covered with 45 x 50 mm glass cover slips (Erie Scientific, Portmouth, NH). The agar was allowed to solidify

(approximately 20 minutes) and the plates incubated at 37°C, 5% CO_2 for three hours, after which the plaques were enumerated.

Dexamethasone 21-phosphate (DEX) is a well-known immunosuppressant and was used for CTL positive control experiments. DEX (Sigma, St. Louis, MO) solutions were prepared in an aqueous medium. Animals were dosed by oral gavage for five consecutive days prior to euthanasia for a final dose of 50 mg/kg for the in vitro sensitization or for 10 consecutive days following in vivo immunization for a final dose of 50 mg/kg. Euthanasia, removal and processing of spleen cells, and the ^{51}Cr release assay were performed for both in vivo and in vitro CTL experiments as described above.

All data were analyzed using Dunnett's multiple comparison t-test (Dunnett, 1955) with a $p<0.05$ considered significant.

VII. RESULTS AND DISCUSSION

Exposure to TCDD had no effect on CTL generation at any dose in this study. Single and weekly exposure results were compared, taking into account weekly metabolism of TCDD (using a biological half life for TCDD of 12 days). A single dose of 7.20, 2.40, 0.72, or 0.24 µg TCDD/kg had no significant effect versus controls on either *in vivo* or *in vitro* generated CTL response at any E:T ratio (see figures 1 and 2). Likewise, four weekly exposures to TCDD did not significantly affect the CTL response versus contols at levels equal to 3.0, 1.0, 0.30, and 0.10 µg TCDD/kg/week or 0.30, 0.10, 0.03, and 0.01 µg/kg/week (see figures 3-6). Due to the amount of scatter in the data, the *in vitro* generated CTL experiment following four weekly exposuresd to TCDD at the higher dose level of 0.1 - 3.0 µg/kg/week was repeated. The quality of the data improved while there was no change in CTL response (see figure 7).

Organ weight effects were observed, as expected. Liver weight was significantly increased and thymus weight decreased at TCDD doses of 3.0 and 1.0 µg/kg/week. Liver to body weight ratios were increased at doses from 0.10 to 3.0 µg TCDD/kg/week; thymus to body weight ratio was decreased at 3.0 µg TCDD/kg/week (see figure 8). This weight effect was not evident when the experiment was repeated as mentioned above (as in figure 7); here no weight effects were detected. No weight changes were observed in the liver, spleen or thymus of mice dosed for four weeks at

or below 0.30 µg/kg/week (see figure 9). A single dose of 7.20 or 2.40 µg TCDD/kg resulted in an increased liver to body weight ratio but no alterations in spleen or thymus weights were observed (see figure 10).

The positive control experiments using DEX were successful; the CTL assay response was shown to be indicative of immunosuppression. Both in vivo and in vitro generated CTL responses were significantly lower than controls (see figures 11 and 12).

As noted earlier, TCDD is expected to suppress the PFC response beginning at around 1.0 µg/kg. Both the number of PFC per spleen and PFC per $1x10^6$ cells were significantly decreased in mice dosed for four weeks at 3.0 and 1.0 µg TCDD/kg/week (see figures 13 and 14).

The results of this study do not agree with the previously published data of Clark et al. (1981). They demonstrated suppression of in vivo generated CTL responses following four weekly TCDD doses of 40, 4.0, and 0.4 µg/kg. The in vitro generated CTL was suppressed in their lab at four weekly doses of 0.4, 0.04, and 0.004 µg/kg (400, 40, and 4 ng/kg). The data presented here suggest that CTL function is not affected at doses of TCDD upto 3.0 µg/kg/week.

The lytic unit, defined by Bryant et al., (1992) as the number of effector cells required to lyse a specified percentage of target cells, has become the primary method expressing CTL lytic activity. Differences in lytic activity across a range of E:T ratios are most often not constant; the lytic unit, therefore, provides some means for standardizing percent specific lysis. Usually, the reference lysis level is 20% and the results reported as lytic units per a specified number of effector cells, often $1x10^7$ cells (Bryant et al., 1992).

Clark et al. (1981) reported their data in terms of lytic units; the reference lysis percentage used was 50%. To determine if massaging the data could in some way help account for their results, the CTL data presented here was converted to lytic units and analyzed, using a computer program written for Clinical Immunolgy Services, Frederick, MD. While the data in Fig. 15 suggest a dose-related decrease in lytic units with increasing TCDD dose, TCDD dosed mice did not have significantly reduced CTL activity. Furthermore, no significant difference was found in the number of lytic units required to lyse 20% of the target population in the other in vivo or the in vitro generated assays (see figures 16-18).

There exists an inherent problem in attempting to prove data false. Unfortunately, exact replication of Clark et al.'s 1981 work was an impossibility. For example, Clark et al. (1981) used male C57Bl/6J mice for their study. Female mice of the same strain were used here, as they are more easily housed together due to their lack of fighting behavior. Though aggressive behavior may alter immune responses through stress-induced immunosuppression and thereby influence the immune response to chemical exposure, it is unlikely that sex differences would account for such a large discrepancy as found here. Also, Clark reported maximal response to alloantigen administered in vivo at around six days. This defies expected immunologic kinetics for the generation of CTL response. The response typically peaks at 10-12 days following viable tumor injection. For this reason, the CTL response against both viable and non-viable P-815 cells was evaluated at six days and at eleven days (see figures 19 and 20). Very poor responses were mounted against either viable or non-viable tumor cells,

administered ip, at six days. A strong response, however, was mounted against only viable tumor cells at eleven days. This time difference could be implicated in the vastly different results of the two studies. Finally, Clark et al. found that thymocytes from treated animals could significantly suppress the CTL activity of both naive and treated splenocytes in culture. Addition of treated thymocytes to in vitro cultures could help account for the exquisite sensitivity demonstrated only in Clark's work.

VIII. Table 1.

LD_{50} for Different Species

Species	LD_{50} (µg/kg)	Reference
guinea pig	2	Neal(1985)
monkey	50	"
adult rat	60	"
weanling rat	25	"
dog	100	Tschirley(1986)
C57B1/6 mouse	132	Neal(1985)
DBA/2 mouse	620	"
B6D2F1 mouse	132	Vecchi(1983)
rabbit	275	Tschirley(1986)
frog	1000	"
hamster	5000	"

Fig. 1 THE EFFECT OF TCDD ON In vivo GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY (single dose)

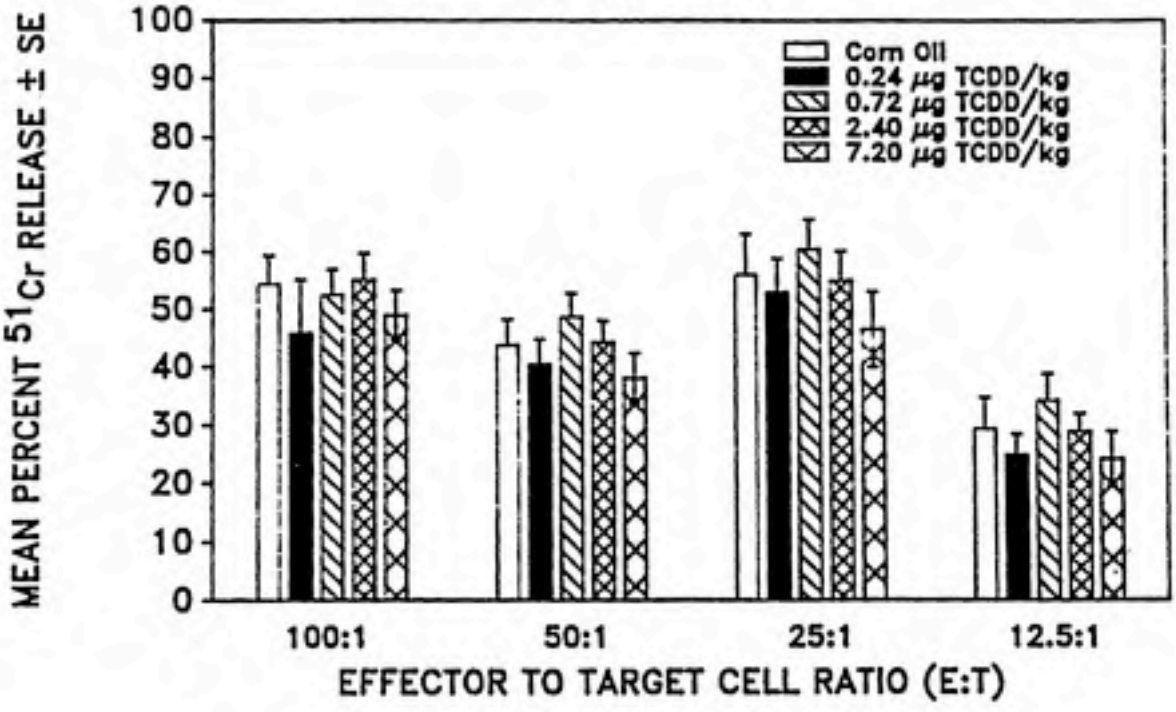


Fig 2. THE EFFECT OF TCDD ON In vitro GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY (single dose)

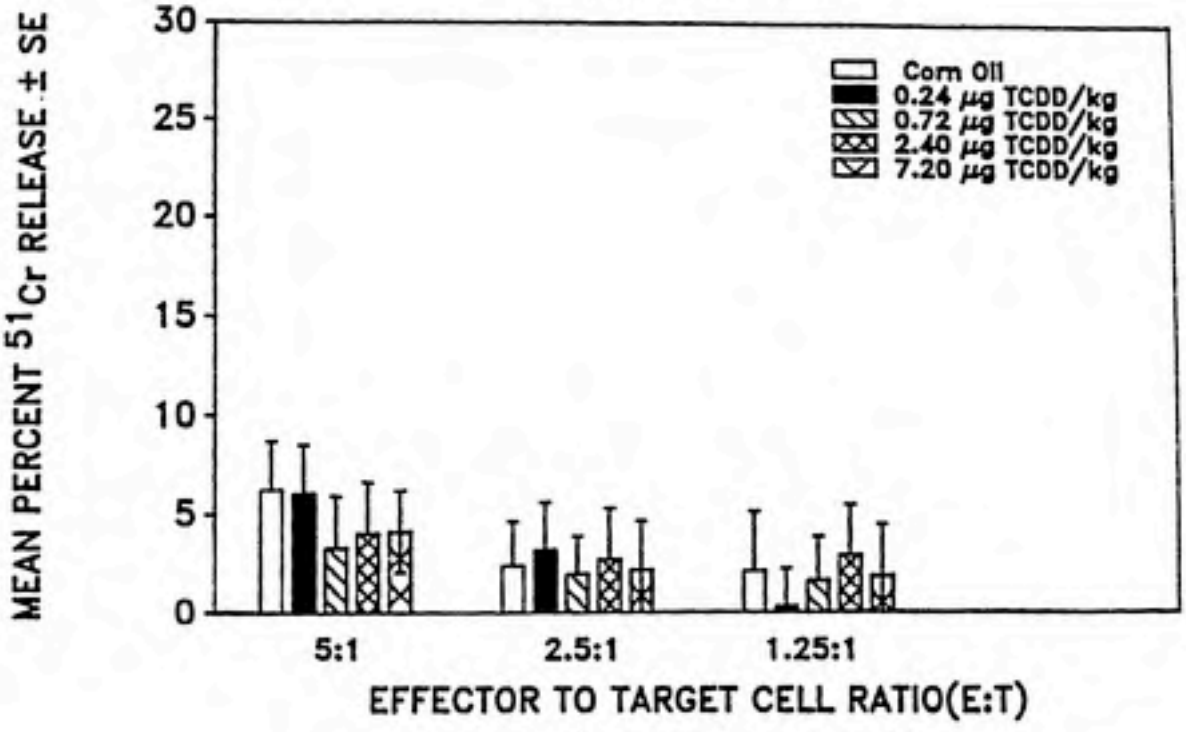


Fig.3 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON in vivo GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

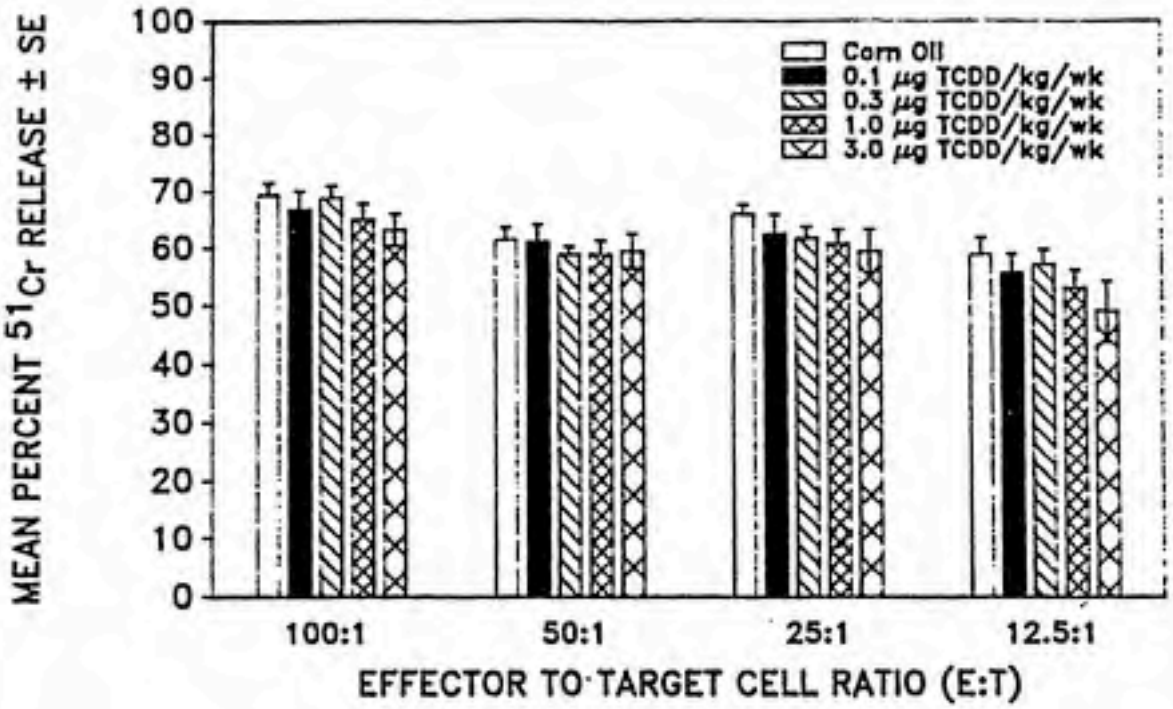


Fig. 4 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON in vivo GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

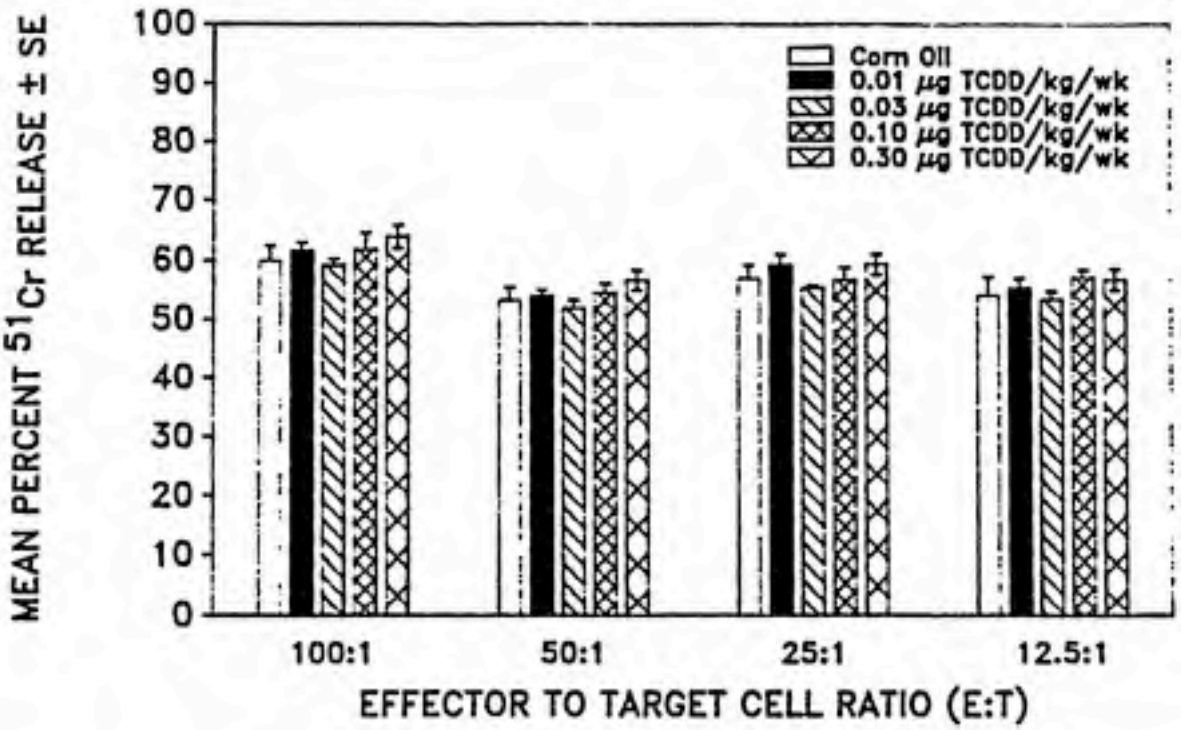


Fig. 5 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON
in vitro GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

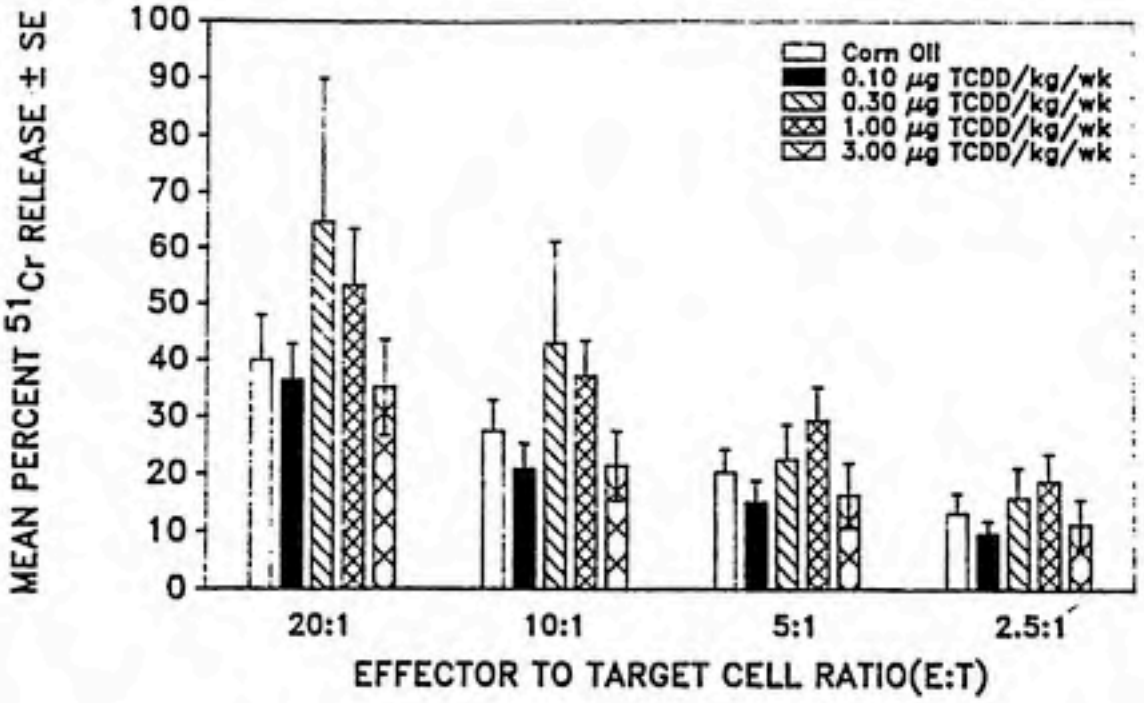


Fig.6 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON
in vitro GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

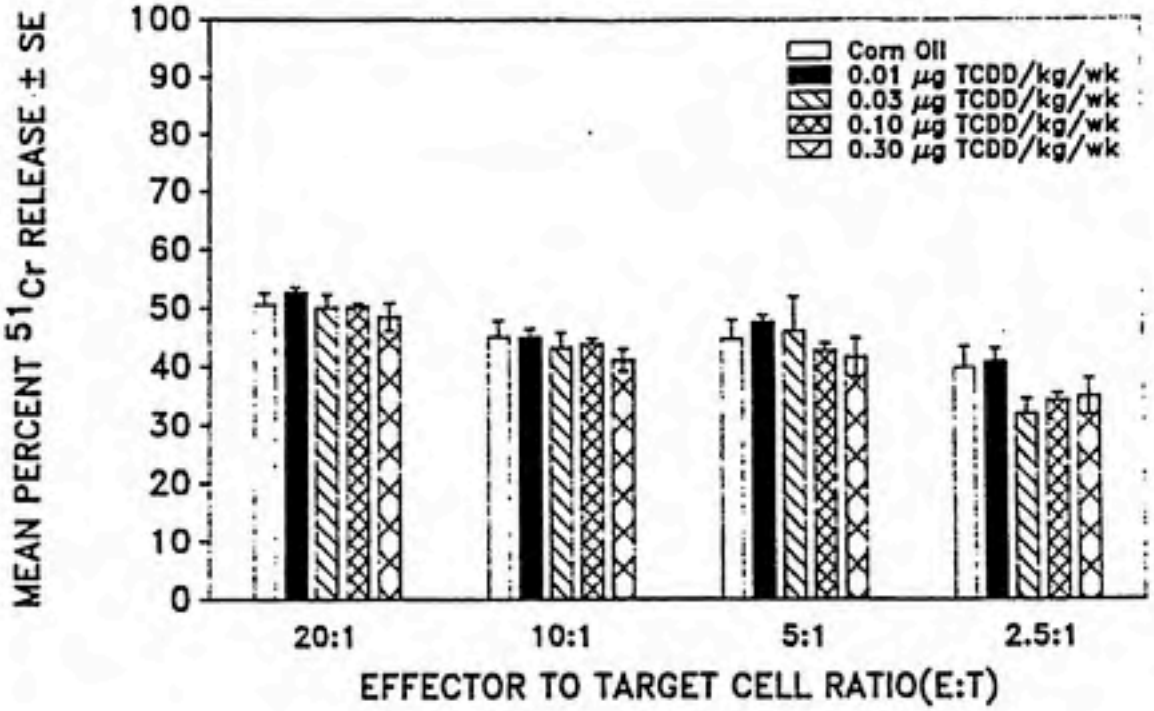


Fig. 7 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON In vitro GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

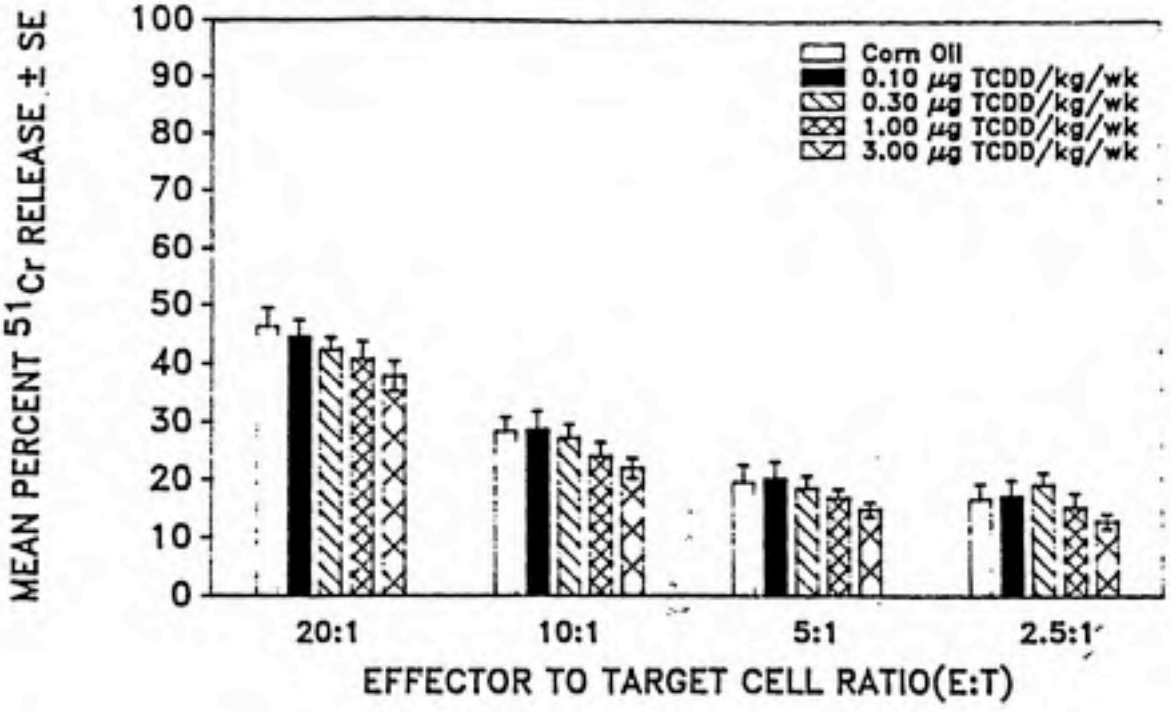


Fig. 8 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON ORGAN WEIGHT

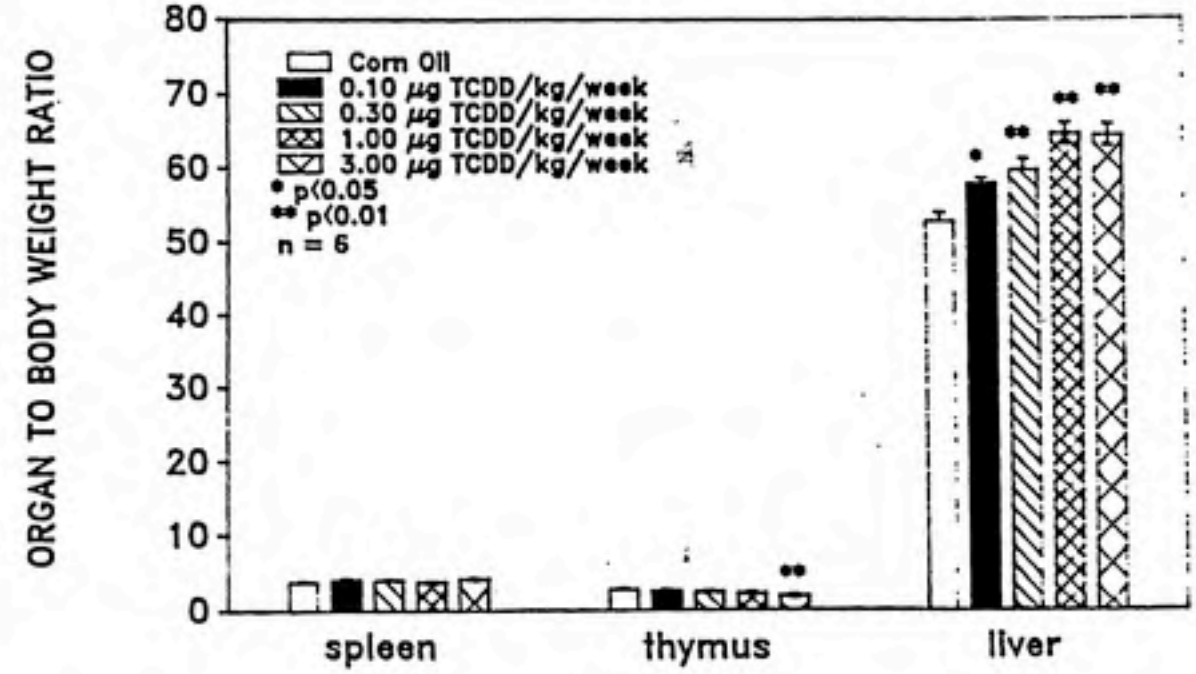


Fig. 9 THE EFFECT OF 4 WEEKLY EXPOSURES TO TCDD ON ORGAN WEIGHT

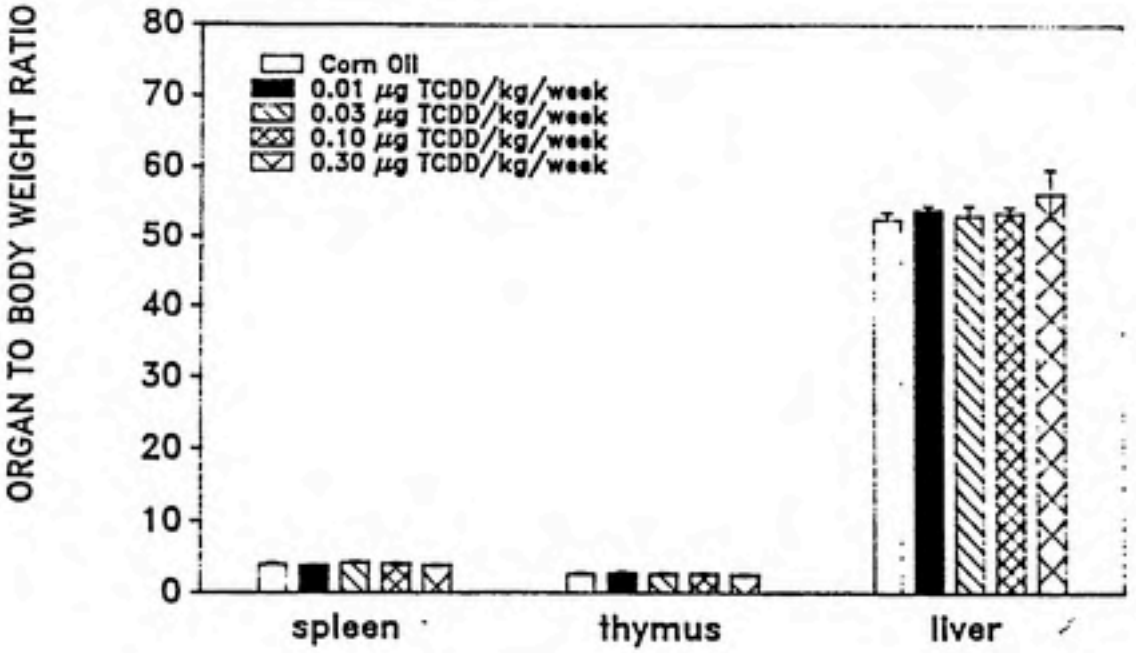


Fig. 10 THE EFFECT OF A SINGLE EXPOSURE TO TCDD ON ORGAN WEIGHT

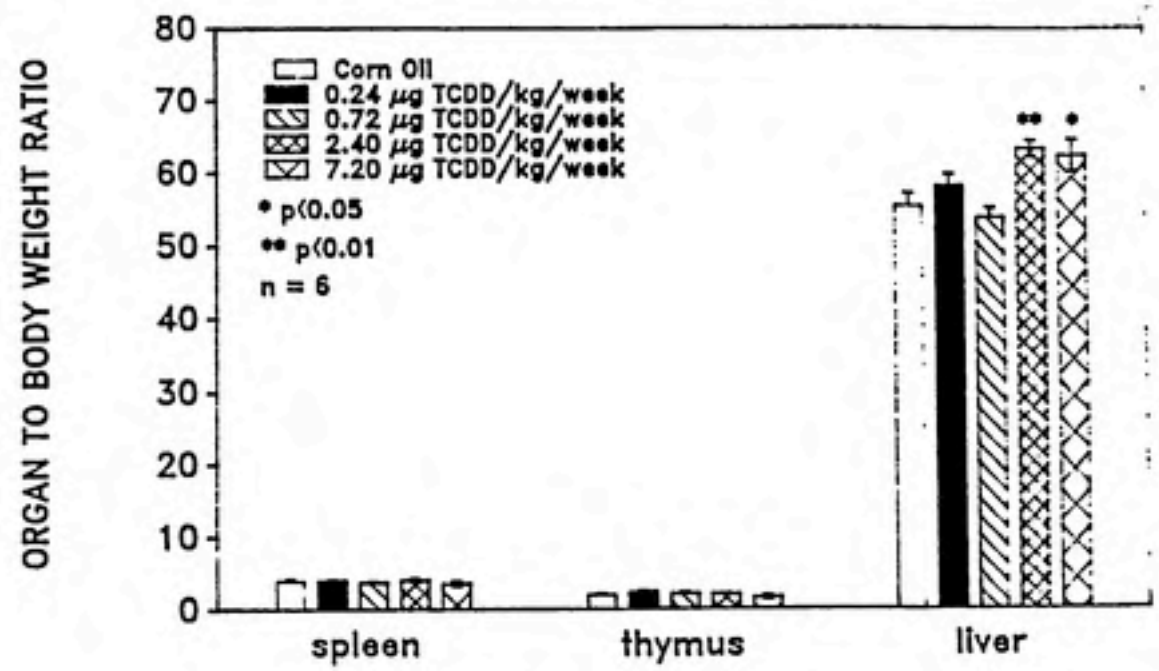


Fig. 11 THE EFFECT OF DEXAMETHASONE ON In vivo GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

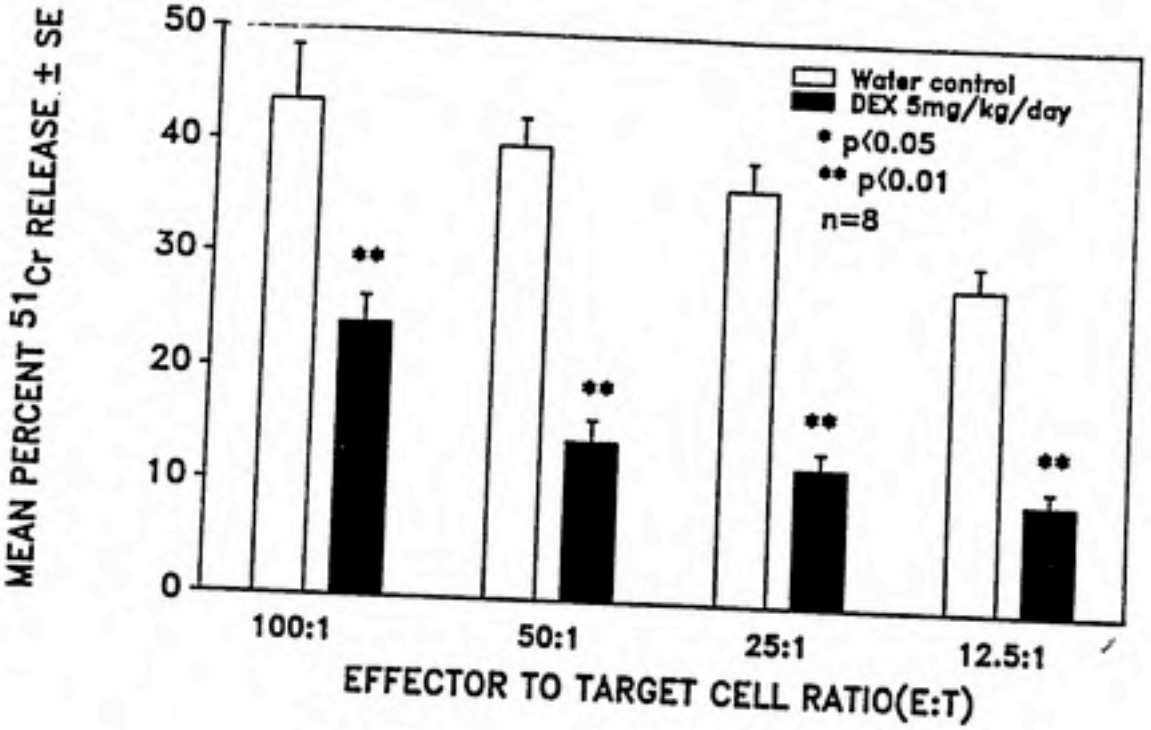


Fig.12 THE EFFECT OF DEXAMETHASONE ON In vitro GENERATED CYTOTOXIC T LYMPHOCYTE ACTIVITY

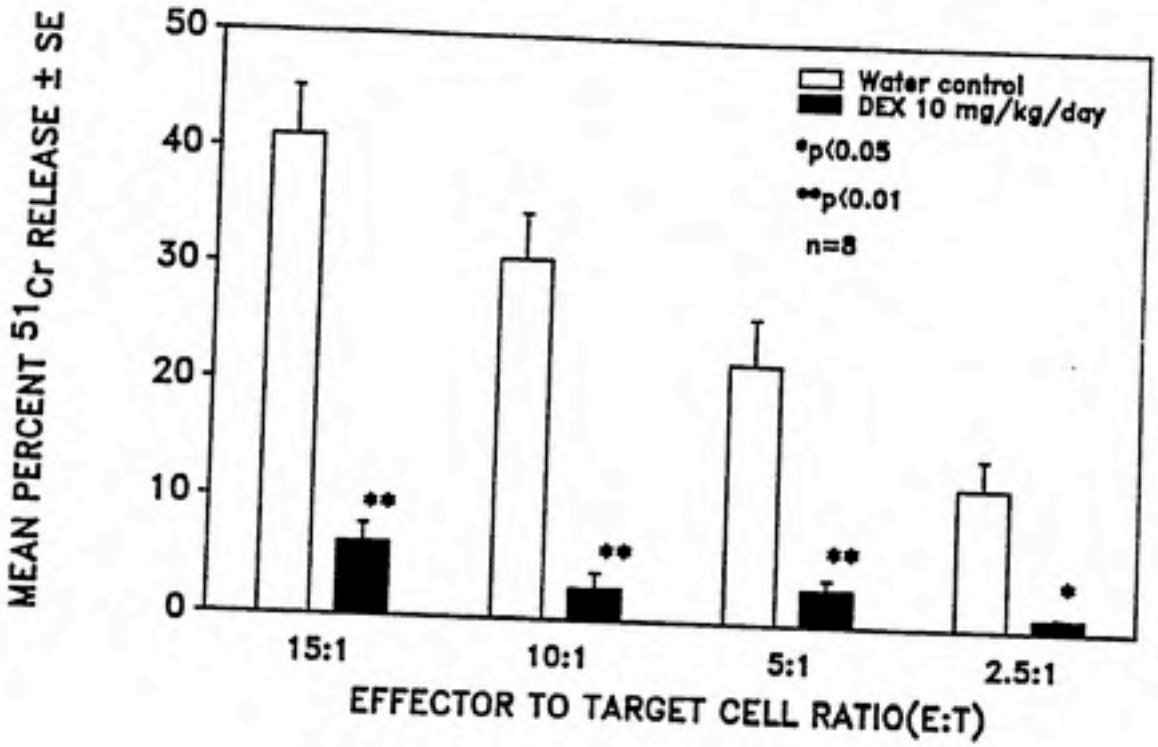


Fig. 13 THE EFFECT OF TCDD ON THE PFC RESPONSE TO SHEEP RED BLOOD CELLS

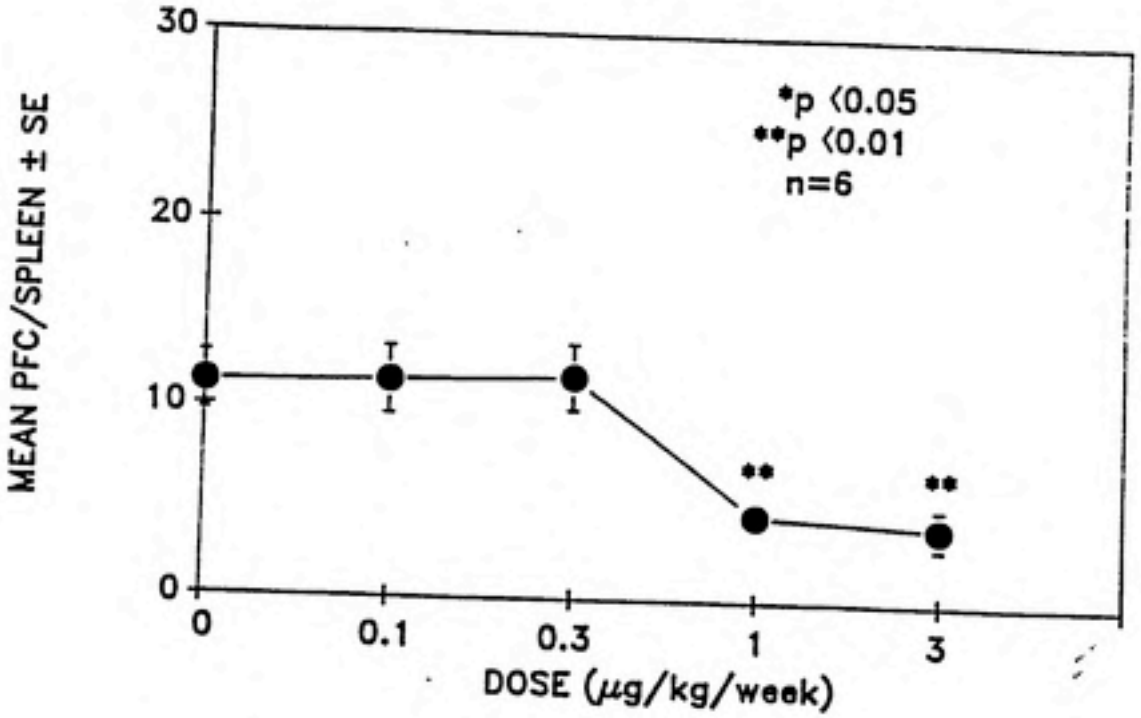


Fig. 14 THE EFFECT OF TCDD ON THE PFC RESPONSE TO SHEEP RED BLOOD CELLS

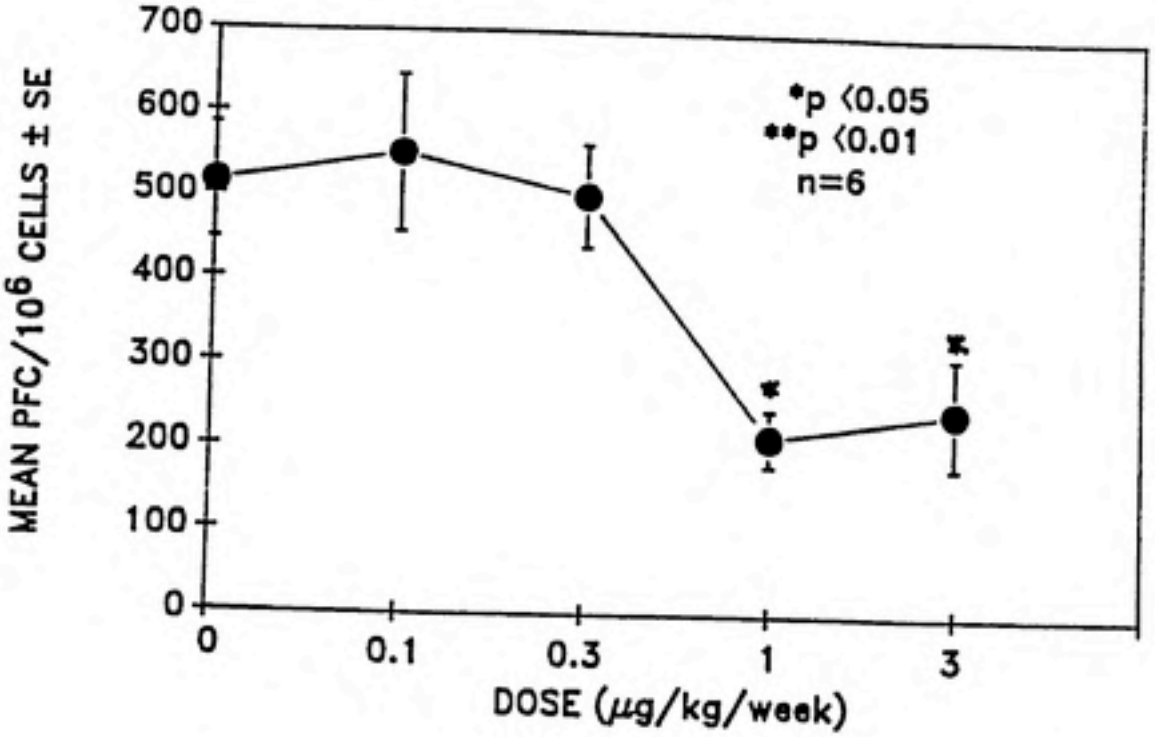


Fig. 15 THE EFFECT OF TCDD ON LYTIC UNITS PER 10^7 CELLS
(In vivo generated CTL)

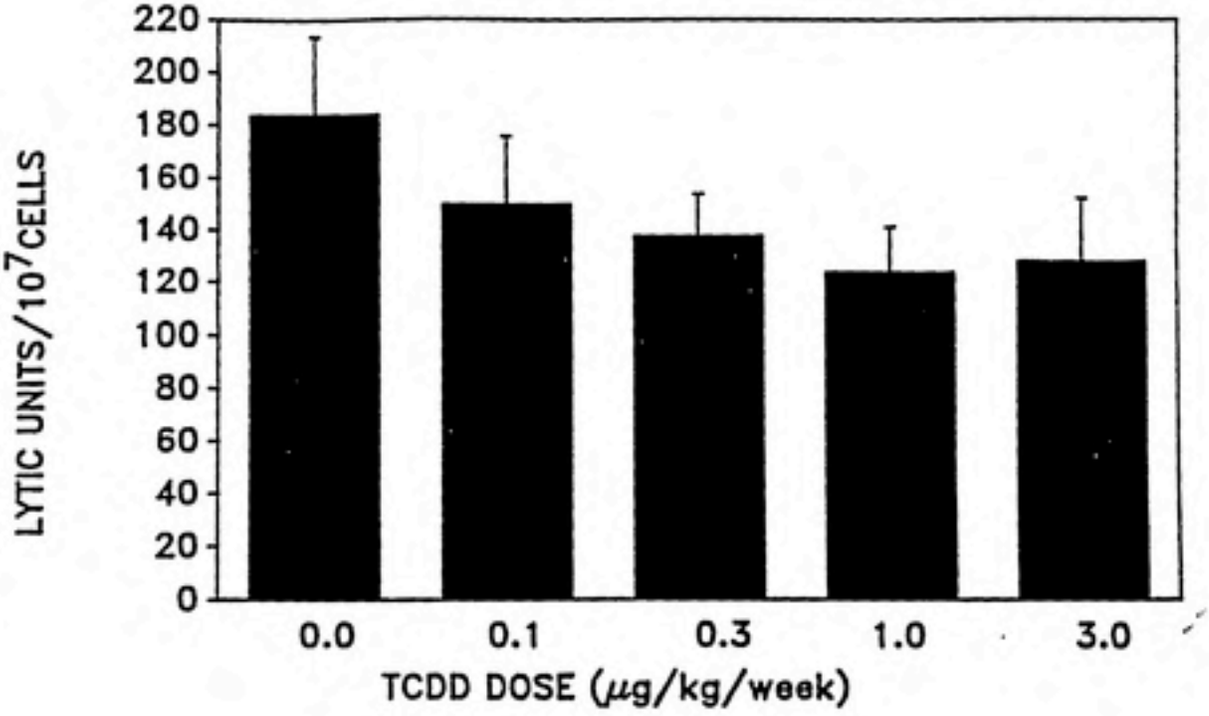


Fig. 16 THE EFFECT OF TCDD ON LYTIC UNITS PER 10^7 CELLS
(In vivo generated CTL)

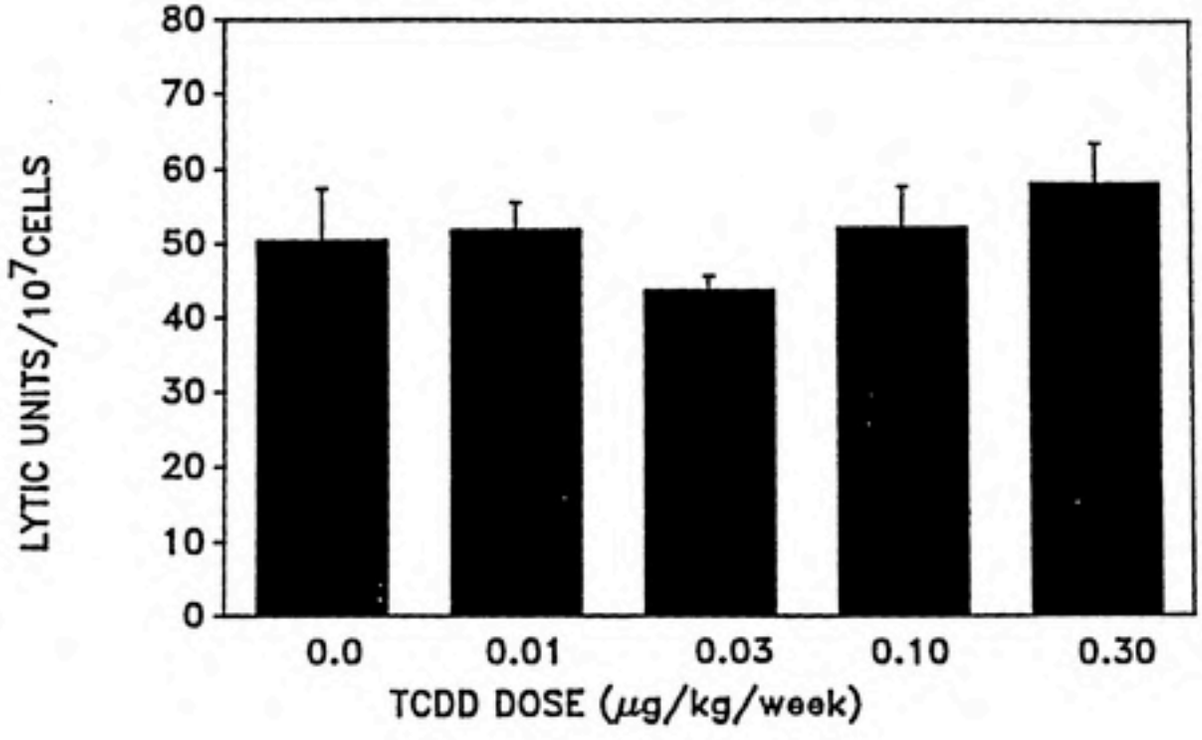


Fig. 17 THE EFFECT OF TCDD ON LYTIC UNITS PER 10^7 CELLS
(in vitro generated CTL)

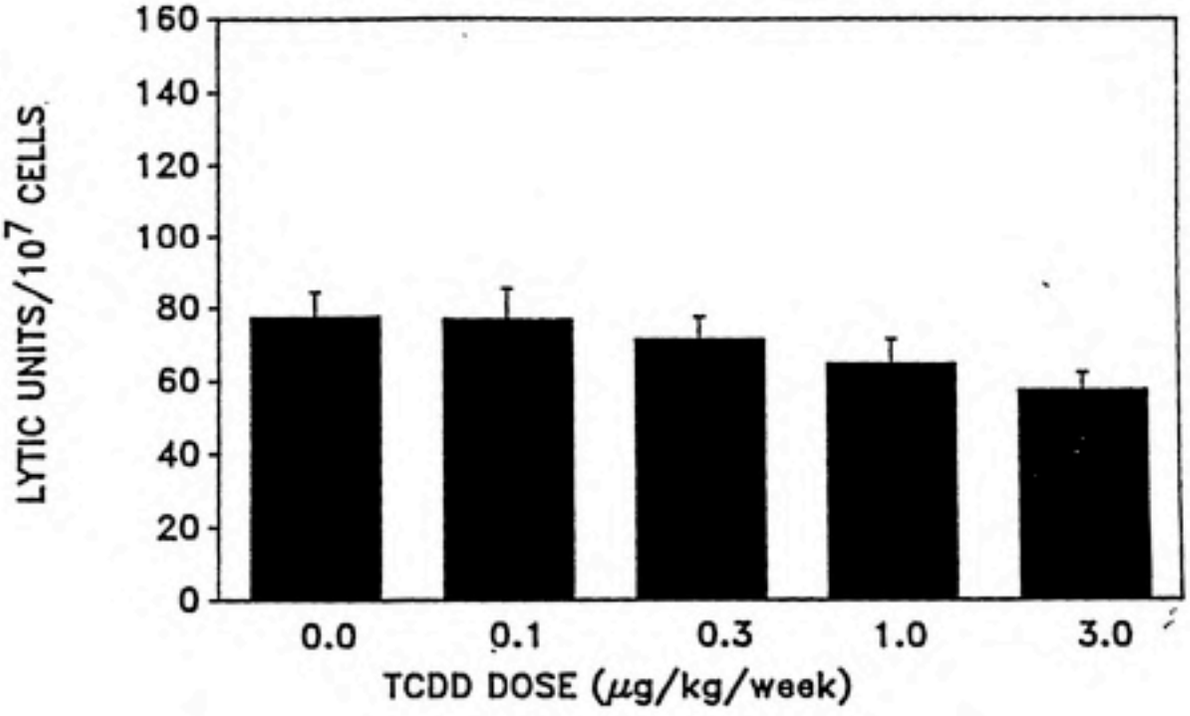


Fig. 18 THE EFFECT OF TCDD ON LYTIC UNITS PER 10^7 CELLS
(in vitro generated CTL)

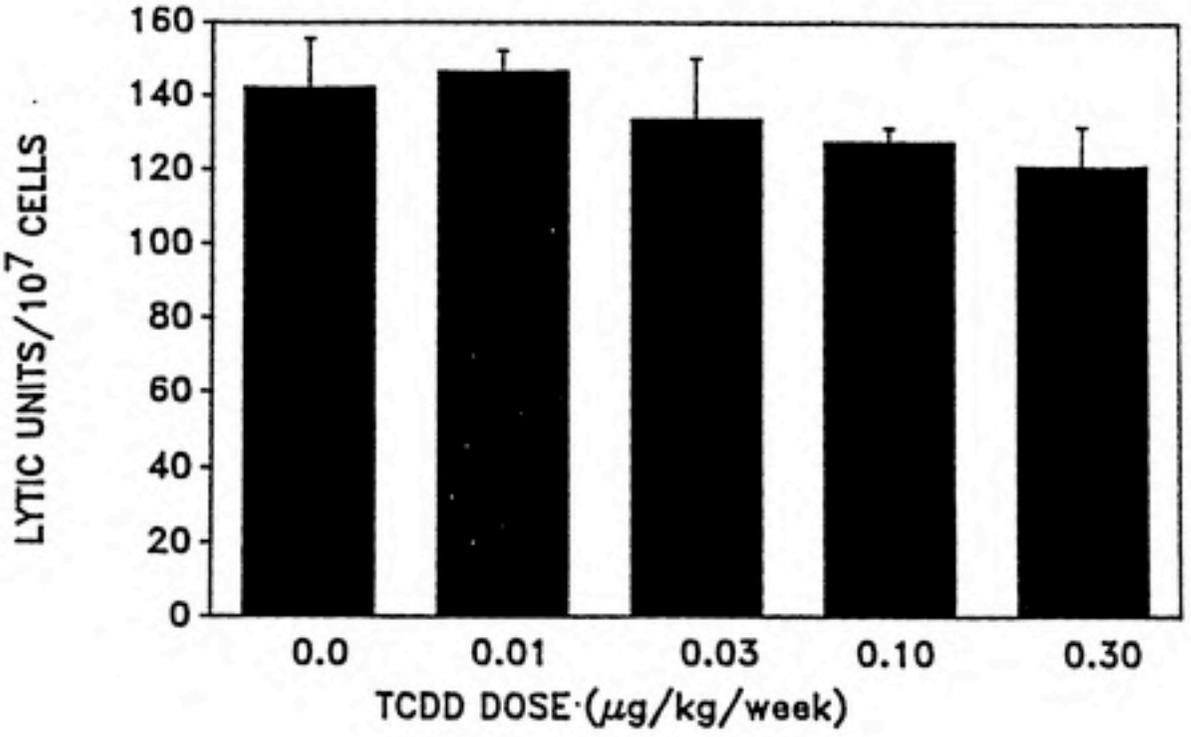


Fig. 19 COMPARISON OF IMMUNIZATION METHOD
(In vivo)
6 DAYS

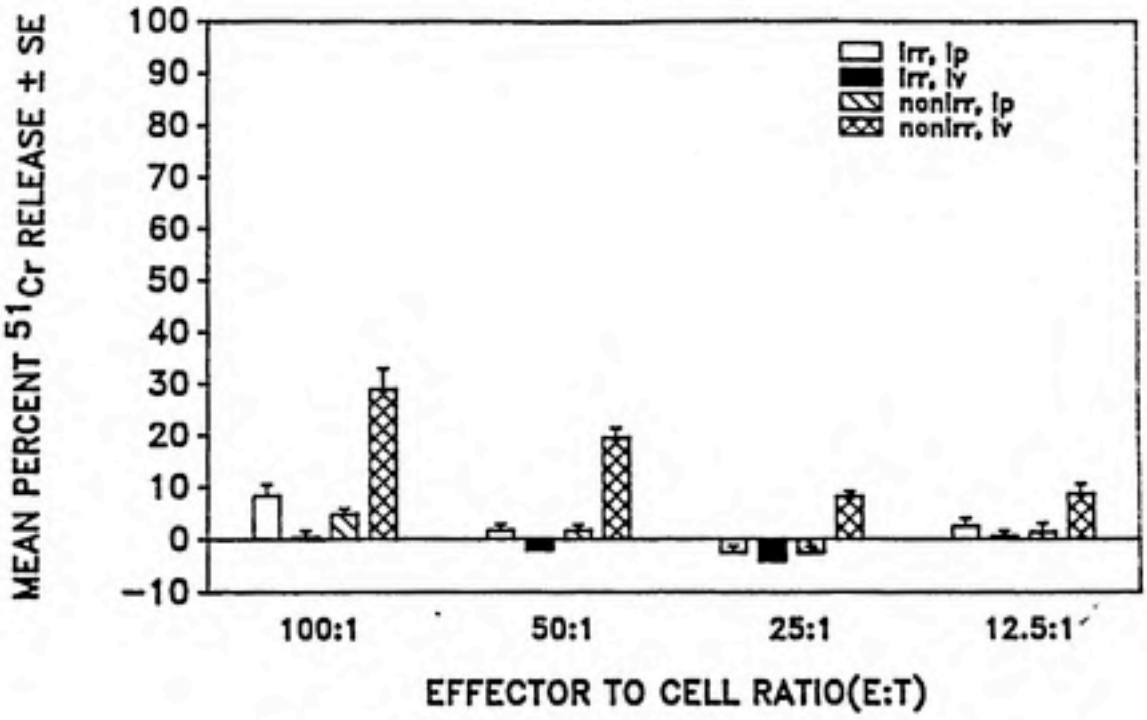
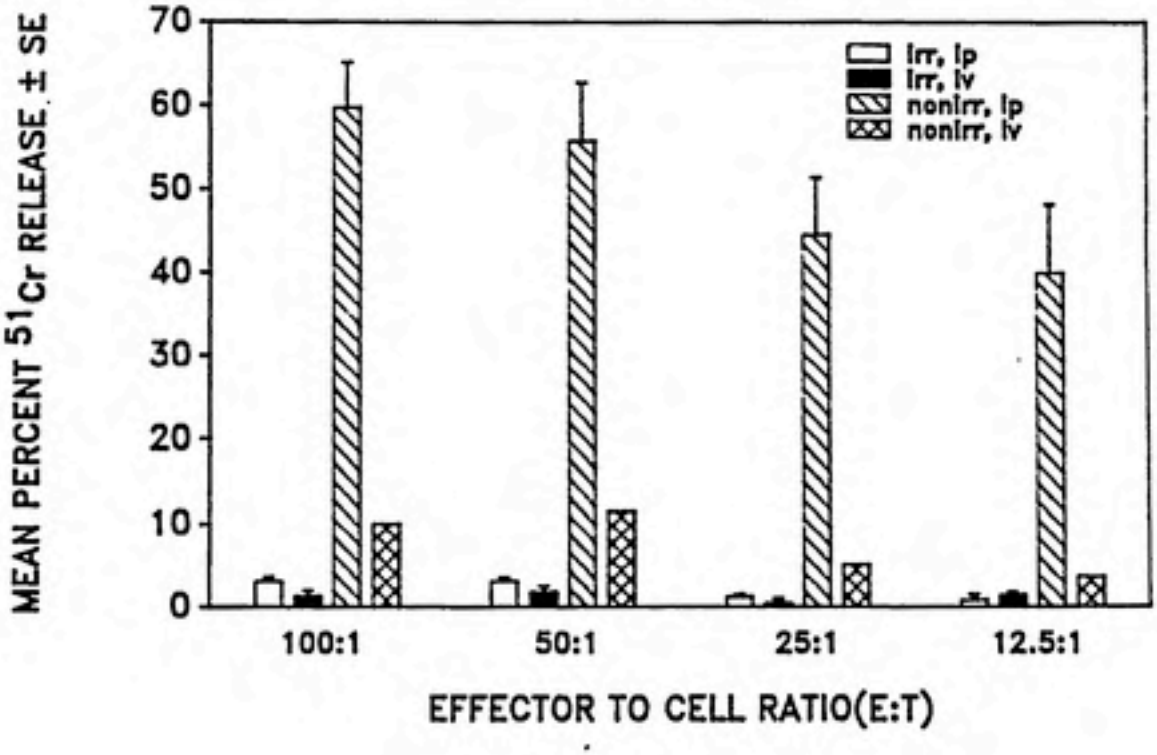


Fig. 20 COMPARISON OF IMMUNIZATION METHOD
(In vivo)
11 DAYS



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